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Maternal high-fat intake during pregnancy programs metabolic-syndrome-related phenotypes through liver mitochondrial DNA copy number and transcriptional activity of liver *PPARGC1A*☆,☆☆

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Abstract

In this study, we contrasted the hypothesis that maternal diet during pregnancy has an impact on fetal metabolic programming through changes in liver mitochondrial DNA (mtDNA) content and transcriptional activity of *Ppargc1a* and that these effects are sex specific.

Methods: Rats were fed either high-fat (HFD) or standard chow diet (SCD) during gestation and lactation. The resulting adult male and female offspring were fed either HFD or SCD for an 18-week period, generating eight experimental groups.

Results: Maternal HFD feeding during pregnancy is associated with a decreased liver mtDNA copy number ($P<.008$). This effect was independent of the offspring sex or diet, and was significantly associated with fatty liver when dams were fed HFD ($P<.05$, adjusted by homeostasis model assessment of insulin resistance, HOMA-IR). We also found that maternal HFD feeding results in a male-specific significant reduction of the liver abundance of *Ppargc1a* mRNA ($P<.004$), which significantly impacted peripheral insulin resistance. Liver expression of *Ppargc1a* was inversely correlated with HOMA-IR ($R=-0.53$, $P<.0003$). Only male offspring exposed to a chronic metabolic insult in adult life were insulin resistant and hyperleptinemic, and showed abnormal liver and abdominal fat accumulation. Liver abundance of *Tfam*, *Nrf1*, *Hnf4a*, *Pepck* and *Ppparg* mRNA was not associated with maternal programming. In conclusion, maternal high-fat diet feeding during pregnancy programs liver mtDNA content and the transcriptional activity of *Ppargc1a*, which strongly modulates, in a sex-specific manner, glucose homeostasis and organ fat accumulation in adult life after exposure to a nutritional insult.

Keywords: Metabolic programming; Mitochondrial DNA; *PGC1a*; *PPARGC1a*; Mitochondrial copy number; NAFLD; High-fat diet; Insulin resistance; Liver; Insulin resistance; Gene expression

1. Introduction

The worldwide prevalence of metabolic syndrome (MS) is alarming, having reached pandemic proportions not only in the adult population [1] but also in children [2]. Many factors are involved in this expanded clinical disorder, including the complex interaction between a genetic predisposing background and the exposure to an environment of overnutrition characterized by diets rich in fat and carbohydrates [3,4]. There is evidence that the close interaction between genes and the environment starts very early in life and can be passed across generations. Because of this phenomenon, the concept of fetal metabolic programming has been proposed, as recently reviewed [5]. This concept emerged after epidemiological observations showed that MS-related diseases, including cardiovascular disease, obesity and diabetes, may be consequences of the 'programming' of the body's structure, physiology and metabolism by the environment during fetal life [6]. Actually, these observations inspired the fetal origin hypothesis of adult diseases, which assumes

Abbreviations: Actb, beta-actin; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; HOMA-IR, homeostasis model assessment of insulin resistance; mtDNA, mitochondrial DNA; MS, metabolic syndrome; nDNA, nuclear DNA; *Hnf4a*, hepatocyte nuclear factor 4, alpha gene; *Nrf1*, nuclear respiratory factor-1 gene; *PPARGC1A*/*Ppargc1a*, human/ rat peroxisome proliferator-activated receptor gamma coactivator-1 alpha gene; *Pepck*, phosphoenolpyruvate carboxykinase 1 gene; *Pparg*, peroxisome proliferator-activated receptor gamma gene; *Ppia*, peptidyl prolyl isomerase A (cyclophilin A) gene; *Tfam*, mitochondrial transcription factor A gene.

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that fetal undernutrition in middle to late gestation not only leads to disproportionate fetal growth but also programs long-term changes in physiology and metabolism [7]. Interestingly, recent evidence from animal and human studies has shown that the implications of early nutrition programming are not limited to fetal undernutrition. On the contrary, the increased prevalence of obesity-related diseases observed in the last decades supports a critical role of the opposite fetal scenario characterized by overnutrition. In fact, animal studies have shown that rats that underwent either a high-carbohydrate [8] or a high-fat (HFD) [9] dietary modification during pregnancy are able to transmit the MS-related phenotypes to their offspring, thus establishing a generational effect.

Programming studies mostly focus on changes in target gene expression that modify the phenotype in the progeny. Recent evidence from experimental animals has shown that mitochondrial dysfunction may be a long-term consequence of a poor nutritional environment during early life [10–12]; similarly, exposure to a maternal diet rich in animal fat is associated with altered mitochondrial gene expression [13]. These studies opened the question on the putative role of nutritional mitochondrial programming. We have reported that both extremes of neonatal birth weight are associated with decreased umbilical cord mitochondrial DNA (mtDNA) content [14]. Furthermore, in a recently published human study that included patients with all features of MS, including nonalcoholic fatty liver disease (NAFLD, the hepatic manifestation of the MS), we observed that both the liver transcriptional activity of the peroxisome proliferator-activated receptor gamma coactivator-1 alpha (*PPARGC1A*) and a change in the liver mtDNA content were associated with peripheral insulin resistance [15]. Hence, we supported the role of the fatty liver as a strong modifier of and/or a principal player in the natural history of MS-related diseases.

In view of all the evidence described and considering the strong influence of the maternal environment during fetal life on modeling the metabolic profile of the progeny, we hypothesized that maternal high-fat feeding during pregnancy is associated with a programming effect on the liver abundance of *Ppargc1a* mRNA, predisposing the offspring to develop insulin resistance and MS-related phenotypes when they are exposed to a metabolic insult in later life. In addition, we hypothesized that maternal programming modulates liver mtDNA content.

To address these hypotheses, we measured the liver mtDNA copy number in the adult offspring of dams fed HFD during gestation and lactation. We additionally examined the liver mRNA abundance of nuclear genes involved in the regulation of mtDNA transcription and replication, including *Ppargc1a*, also known as *PGC1α*, a master transcriptional coactivator involved in the regulation of mitochondrial biogenesis. Finally, we also aimed to test whether the effect on fetal programming was sex specific, as we had found preliminary evidence that this may be the case [16].

2. Materials and methods

2.1. Animals and study design

Twelve-week-old female Wistar rats of first-order parity and weighing 228±10 g were randomly assigned to either *ad libitum* HFD solid diet (40% wt/wt bovine and porcine fat added to the standard chow) [17] or standard chow diet (SCD). The HFD provided 5340–5460 (kcal/kg) vs. the SCD's 2900–3100 (kcal/kg), and 13.8% of proteins vs. 23%, respectively. The amount of proteins in the HFD was adequate as recommended by nutrition guidelines for rats to be maintained after a rapid growth [18].

Dams were fed 15 days before conception and during gestation and lactation; weight gain and food intake were measured at these periods. Upon birth, pups were sexed, and litter size was noted. To ensure homogeneity of offspring evaluated, within 24 h of birth, all litters studied were adjusted to 9/10 pups per dam; the sex ratio was maintained as close to 1:1 as possible in order to study potential sexual dimorphism. Thus, each experimental group has offspring of two or three mothers; pups continued to be weighed every week.

The offspring at the age of 17 weeks were randomly assigned either *ad libitum* HFD or SCD for an 18-week period, generating eight experimental groups according to male (M) or female (F) sex distribution and maternal HFD or SCD feeding.

All the animals were housed individually, with food and water freely available, and were maintained at room temperature (23°C+1°C) under a 12-h light/dark cycle.

At the completion of the study, the animals were sacrificed by decapitation, and blood samples were collected in tubes containing sodium EDTA and centrifuged; plasma was immediately frozen.

Food was withdrawn from 8:00 a.m. to 4:00 p.m. before the rats were sacrificed, and the intraperitoneal and retroperitoneal fats were measured by weighting them directly.

The liver was quickly snap-frozen and stored at −76°C until gene expression analysis. A portion of each liver was fixed in 10% formalin for histological analysis.

All the animals received humane care, and the studies were conducted according to the regulations for the use and care of experimental animals.

2.2. Biochemical measurements

Serum and sodium EDTA-plasma were obtained by centrifugation and stored at −80°C until needed. Fasting glucose was measured by an automatic biochemical analytical system (Architect, Abbott, Buenos Aires, Argentina). The plasma insulin levels were determined using a commercial quantitative ultrasensitive enzyme-linked immunosorbent assay rat kit according to the manufacturer's instructions (Crystal Chem Inc., Downers Grove, IL, USA). Insulin resistance was calculated using the homeostasis model of assessment (HOMA-IR) index [fasting plasma insulin (μU/ml)×fasting plasma glucose (mmol/L)/22.5].

2.3. Histological analysis of liver tissue

Formalin-fixed liver tissue was processed, and 5-μm-thick paraffin sections were stained with hematoxylin and eosin (H&E). The degree of steatosis was assessed irrespective of the experimental groups as previously described [19] based on the percentage of hepatocytes containing macrovesicular fat droplets: grade 0, no steatosis or steatosis <5%; grade 1, >5% to <33% of hepatocytes containing macrovesicular fat droplets; grade 2, >33% to 66% of hepatocytes containing macrovesicular fat droplets; and grade 3, >66% of hepatocytes containing macrovesicular fat droplets.

2.4. Quantification of mtDNA

We used an assay based on real-time quantitative polymerase chain reaction (PCR) for both nDNA and mtDNA quantification, with SYBR-Green as a fluorescent dye (Invitrogen, Buenos Aires, Argentina). For the detection of liver nDNA, we selected *GAPDH* between the nucleotides 1443 and 1571, and for the detection of mtDNA, we selected the mitochondrial-encoded 16S RNA (*Rnr2*) between the nucleotides 2451 and 2583 [20]. The results were presented as the mitochondrial DNA/nuclear DNA ratio (mtDNA/nDNA). Duplicate amplifications of mitochondrial and nuclear products were performed separately.

Real-time quantitative PCR was carried out in a BioRad iCycler (Bio-Rad Laboratories, Hercules, CA, USA). The calculation of DNA copy number involved extrapolation from the fluorescence readings in the mode of background subtracted from the readings of the BioRad iCycler according to the above-described procedure for gene expression. Melting curve analysis was carried out at the end of each run to confirm the specificity of amplification and the absence of primer dimers. The two target amplicon sequences (mtDNA and nDNA) were visualized in 2% agarose and purified using the Qiagen Qiaex II Gel Extraction Kit (Tecnolab, Buenos Aires, Argentina). The dilutions of the purified amplicons were used as the standard curve to verify the PCR-efficiency values estimated by using the procedure described below.

2.5. RNA preparation and real-time reverse transcriptase (RT)-PCR for quantitative assessment of mRNA expression

Total RNA was prepared from rat livers using the phenol extraction step method, with an additional DNAse digestion step. For RT-PCR, 3 μg of total RNA was reverse-transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (Promega, WI, USA). Real-time PCR was performed for quantitative assessment of mRNA expression in an iCycler thermocycler (BioRad, Hercules, CA, USA) using the fluorescent dye SYBR-Green (Invitrogen, Buenos Aires, Argentina). All the real-time PCRs were run in duplicate, and all the samples of the experimental groups were tested. The relative expression of the target genes' mRNA was normalized to the amount of a housekeeping gene (peptidyl prolyl isomerase A, Ppia, also termed as cyclophilin A) to carry out comparisons between the groups. Cyclophilin was found to be the most stable reference gene for testing liver mRNA expression among other housekeeping genes tested before starting the experiment [β-actin, TATA box binding protein, and glyceraldehyde-3-phosphate dehydrogenase (Gapdh)]. The levels of mRNA were expressed as the ratio of the estimated amount of the target gene relative to the Ppia mRNA levels using fluorescence threshold cycle values calculated for each sample, and the estimated efficiency of the PCR for each product was expressed as the average of each sample efficiency value obtained [21].